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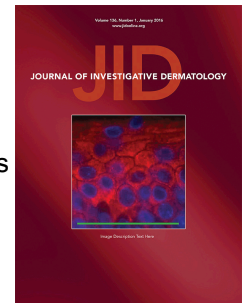
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Congenital anonychia and uncombable hair syndrome: co-inheritance of homozygous mutations in *RSPO4* and *PADI3*

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Short title: Congenital anonychia and uncombable hair syndrome

Abbreviations used: ED, ectodermal dysplasia; PADI3, peptidyl arginine deiminase 3; RSPO4, R-Spondin 4; WES, whole exome sequencing; HF, hair follicle; WT, wild-type.

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TO THE EDITOR,

Ectodermal dysplasia (ED) comprises a large, heterogeneous group of inherited disorders defined by developmental defects in two or more tissues derived from embryonic ectoderm, including the skin, its appendages (i.e., hair follicles, eccrine glands, sebaceous glands, and nails) and teeth (Itin, 2014). The marked heterogeneity of ED hinders accurate genotype-phenotype correlation, although application of next generation sequencing has added further insights (Lin et al., 2012; Petrof et al., 2014; Raykova et al., 2014). Clinical assessment and accurate diagnosis are further compounded by observations that congenital nail or hair disorders may occur as isolated phenomena (Khan et al., 2015; Shimomura, 2012). Here, we investigate a case presenting with congenital anonychia and uncombable sparse hair, and use whole exome sequencing (WES) to demonstrate that the phenotype

results from two separate autosomal recessive disorders rather than a single variant of ED. Permission from the subject's guardian (and also the guardians of the other children mentioned in this report) was given for publication of clinical images and data.

The proband, a 4-year-old Kuwaiti boy born to consanguineous parents, had complete absence of all 20 nails from birth (Figure 1a). In addition, he had somewhat sparse scalp hair that was slow to grow, of wavy texture, and difficult to comb (Figure 1b). There was no other abnormality apart from a congenital squint. The proband is shown as individual IV-3 in the pedigree (Figure 1c). Clinical abnormalities were also present in his cousins (individuals IV-5, IV-6, and IV-7 in Figure 1c), again born to consanguineous parents. Those siblings had a similar absence of all 20 nails but lacked any hair abnormalities (Figure 1d). The clinical conundrum was whether the proband with anonychia and hair abnormalities had a different ED disorder or whether he might have two separate autosomal recessive ectodermal conditions. With regard to the anonychia, biallelic mutations in *RSPO4* (OMIM 610573) have been identified in autosomal recessive congenital anonychia/hyponychia (Bergmann et al., 2006; Blaydon et al., 2006). The encoded protein, R-spondin 4, is expressed in nail mesenchyme and acts as an activator of Wnt/ β -catenin signaling, notably for nails (Blaydon et al., 2006). Following written informed consent and institutional ethics approval, we Sanger-sequenced *RSPO4*, using published methods (Blaydon et al., 2006), and identified a homozygous donor splice-site mutation, IVS1+1G>A, in all anonychic individuals, including the proband and his three cousins (Figure 2a). To date, 18 mutations in *RSPO4* have been identified in isolated congenital anonychia/hyponychia (Khan et al., 2015). The particular splice-site mutation in our pedigree previously has been reported as pathogenic (Blaydon et al., 2006).

To delineate the genetic basis of the hair abnormality, WES was performed on the Illumina NextSeq 500 platform using genomic DNA samples from the proband and both his unaffected parents (trio). Using various *in silico* pathogenicity prediction tools, including SIFT, PolyPhen-2, MutationTaster, CADD and DANN, and models of autosomal recessive or *de novo* dominant inheritance, we identified 11 possible autosomal recessive mutations and 3 *de novo* dominant changes (Tables S1 and S2). A nonsynonymous homozygous mutation in *PADI3* (peptidyl arginine deiminase, type III) (c.1372C>A; p.Pro458Thr) was thought to be the most likely causative finding (SIFT: 0; Polyphen-2: 0.989; MutationTaster: 0.997; CADD: 26.3; DANN: 0.998). This mutation is very rare in the general population (25 heterozygous allele counts among 121406 alleles in the ExAC Browser, <http://exac.broadinstitute.org/>) with no homozygotes. Sanger sequencing confirmed this homozygous missense mutation in *PADI3* in the proband's DNA. Only the proband was homozygous; the other unaffected family members were wild-type (WT) or heterozygous carriers (Figure 2b, c).

Peptidylarginine deiminases (protein name abbreviated to PAD; gene name abbreviated to *PADI*) are Ca^{2+} -dependent enzymes responsible for the formation of protein-bound citrulline, first detected in the hair follicles (HF) of the guinea-pig (Rogers and Taylor, 1977). In humans, five different isoforms (PAD1–4 and PAD6) have been identified in various tissues and all are clustered on 1p35–36 (Vossenaar et al., 2003). PAD-3 is involved in deiminating trichohyalin in the HF medulla and Henle layer (Nachat et al., 2005). The relevance of PAD-3 to hair biology has been further emphasized by the recent discovery of biallelic *PADI3* mutations in 9 cases/families with uncombable hair syndrome (Basmanav et al., in press). The mutations reported comprise three missense variants: c.335T>A (p.Leu112His), c.881C>T (p.Ala294Val), and c.1813C>A (p.Pro605Thr) (Figure 2d), either as homozygous or compound heterozygous findings (Basmanav et al., in press). Our

homozygous amino acid substitution, p.Pro458Thr, occurs in the PAD domain, which is important for protein-arginine deiminase activity and calcium ion binding.

To examine the functional significance of p.Pro458Thr, we engineered a mutant construct by performing site-specific mutagenesis on the vector that was previously used to study the other *PADI3* mutations (see Basmanav et al., in press, for full details). The construct contains the WT *PADI3* sequence (1995 bp) inserted into the pcDNA3.1 V5/His TOPO TA vector (Invitrogen). Mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis kit according to the manufacturer's instructions (Agilent Technologies) with verification by Sanger sequencing. Western blotting of extracts from HaCaT cells transfected with the WT and mutant *PADI3* constructs showed a 4.7-fold reduction in *PADI3* mutant protein (Figure 2e). Immunofluorescence microscopy of HaCaT cells revealed stark differences in the distribution of WT and mutant proteins. While WT *PADI3* was homogeneously distributed in the cytosol, the mutant protein formed aggregates, which were diffuse in the cytoplasm and particularly evident around the nucleus (Figure 2f). These results were similar to the cell pathology shown for the reported p.Ala294Val and p.Pro605Thr constructs, although our mutant construct (p.Pro458Thr) was associated with more aggregation than was seen for the previously assessed mutant p.Leu112His (Basmanav et al., in press). In contrast, no aggregates at all were observed in cells transfected with WT constructs.

The hair phenotype in our proband was also uncombable, due to its wavy textural change, but was predominantly sparse and slow growing, thus differing slightly from all but one of the other reported *PADI3* cases (Basmanav et al., in press) and underscoring the range of hair pathologies that can result from *PADI3* mutations. Our case also highlights the value of WES in identifying co-inheritance of two distinct autosomal recessive conditions in

consanguineous pedigrees which, for one individual herein, jointly gave rise to an ectodermal dysplasia phenotype.

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Figure legends

Figure 1. Nail and hair abnormalities in the proband and extended pedigree. (a) Complete absence of all 20 nails in the proband; (b) The proband has somewhat sparse wavy scalp hair that is slow to grow and difficult to comb; (c) The proband (IV-3, arrow) is the third child of consanguineous parents with one unaffected sister and one unaffected brother. Three cousins (IV-5, IV-6 and IV-7) also have anonychia but no hair abnormalities. (d) The cousins of the proband (IV-5, IV-6 and IV-7) manifest anonychia without hair abnormalities.

Figure 2. Co-inheritance of two separate autosomal recessive disorders accounts for the ectodermal dysplasia phenotype (a) Sanger sequencing identifies a recurrent homozygous donor splice-site mutation in *RSPO4* (IVS1+1G>A) in all subjects with anonychia, including the proband and three cousins; (b) Sanger sequencing confirms the proband's homozygous missense mutation in *PADI3* (c.1372C>A; p.Pro458Thr), originally identified by WES trio analysis. The proband is homozygous for the *PADI3* mutation, while other unaffected family members are wild-type or heterozygous carriers; (c) Verification of the mutation by *HpyCH4III* restriction enzyme digestion (New England BioLabs). The mutation c.1372C>A creates a new cut site such that the 452-bp band is cleaved into 300-bp and 152-bp products. For the patient (IV-3) two cleaved bands are present, whereas in carriers (III-3, III-4, and III-8) three discrete bands are seen. For the wild type only the single undigested upper band is visible. NC, negative control; PC, positive control; MW, molecular weight ladder. (d) Schematic illustration of the PAD-3 protein and the mutations identified thus far (new mutation boxed in red); (e) Immunoblot analysis of protein extracts from transiently

transfected HaCaT cells shows a reduced expression of mutant PADI3 protein level compared to wild-type (wt); immunoblotting was performed with anti-V5 antibody. (f) Immunofluorescence analysis in HaCaT cells transiently expressing WT and mutant PADI3 shows the homogeneous cytosolic expression of WT PADI3 whereas the mutant protein is observed in the form of large aggregates (scale bar = 10 μ m).

